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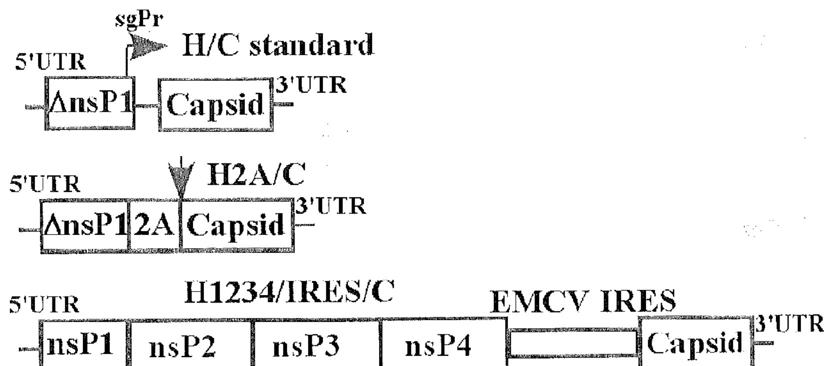
(54) **Title**: METHODS AND COMPOSITIONS FOR PSEUDOINFECTIOUS ALPHAVIRUSES

Fig. 1



(57) **Abstract**: The present invention provides pseudoinfectious alphavirus particles and methods of making them and using them to produce an immune response to an alphavirus in a subject.

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METHODS AND COMPOSITIONS FOR PSEUDOINFECTIOUS ALPHAVIRUSES

STATEMENT OF PRIORITY

This application claims the benefit, under 35 U.S.C. § 119(e) of U.S. Provisional
5 Application Serial No. 61/438,441, filed February 1, 2011, the entire contents of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT SUPPORT

Aspects of this invention were supported by funding under Grant No. R01AI070207
10 from the National Institutes of Health. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to pseudoinfectious alphavirus particles and their use in
15 eliciting an immune response to an alphavirus in a subject.

BACKGROUND OF THE INVENTION

Of all the disease preventative measures devised to date, excluding the sanitation of water, vaccine development has made the greatest contribution to human health. Most of the
20 currently available live attenuated vaccines (LAVs) were developed by serial passage of viruses in tissue culture or chicken embryos, and their attenuated phenotype relies on a very limited number of point mutations, which mainly accumulate in the structural genes. Thus, LAVs induce strong, protective, long-lived immune responses, characterized by a balanced combination of circulating neutralizing antibodies and cellular immunity. However, they
25 demonstrate residual reactogenicity and their reversion to a more pathogenic phenotype during vaccination remains a strong possibility. Inactivated (INVs) and subunit viral vaccines, on the other hand, demonstrate high safety, but induce cellular immunity very inefficiently. They typically require multiple doses to achieve protective immunity, as well as frequent boosters, making the vaccination process lengthy and expensive. In some cases,
30 preparation of samples prior to chemical inactivation also requires high biocontainment conditions.

Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), chikungunya virus and various other alphaviruses represent a serious public health threat. They continuously circulate in different

parts of the world, including South America, Central America and North America. VEEV has a strong potential for use by terrorists and as biological warfare agent. It has been weaponized and can be applied either alone or in combination with other pathogens. VEEV is classified as a category B, select agent. A need still exists for effective antivirals or vaccines against VEEV and other alphavirus infections.

The present invention overcomes previous shortcomings in the art by providing pseudoinfectious alphaviruses (PIV), combining the efficiency of live attenuated vaccines (LAVs), which results from the ability of the PIV genome to replicate and produce subviral particles (SVPs), with the safety of inactivated (INV) viral vaccines, due to the inability of PIV to develop a spreading infection.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a pseudoinfectious alphavirus particle comprising a genome encoding: a) alphavirus nonstructural proteins nsP1-4, b) alphavirus structural proteins E2 and E1, and c) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, whereby binding of RNA to the capsid protein is substantially diminished or eliminated, thereby resulting in production of a noninfectious subviral particle lacking genetic material.

In a further aspect, the present invention provides a noninfectious subviral particle, comprising alphavirus structural proteins E2 and E1 and an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, and lacking genetic material.

Additionally provided herein is a method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of a pseudoinfectious alphavirus of this invention.

Further aspects of this invention include a method of treating and/or preventing an alphavirus infection in a subject, comprising administering to the subject an immunogenic amount of pseudoinfectious alphavirus of this invention.

Also provided herein is a method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing into an alphavirus permissive cell: a) a nucleic acid molecule encoding: i) alphavirus nonstructural proteins nsP1-4, ii) alphavirus structural proteins E2 and E1, and iii) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain; and b) a helper nucleic acid molecule encoding alphavirus RNA-binding competent capsid protein; and

maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

The present invention further provides a method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing a nucleic acid molecule encoding: i) alphavirus nonstructural proteins nsp1-4, ii) alphavirus structural proteins E2 and E1, and iii) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, into an alphavirus permissive cell containing a nucleic acid molecule encoding alphavirus RNA-binding competent capsid protein; and maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

Furthermore, the present invention provides a population of alphavirus particles comprising the pseudoinfectious alphavirus particles produced by a method of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic representation of helper constructs of this invention: H/C standard (top), H2A/C (middle) and H1234/IRES/C bottom, each encoding an RNA packaging-competent alphavirus capsid protein. H/C standard is a helper with an alphavirus subgenomic promoter. H2A/C is a helper that lacks the subgenomic promoter and does not recombine into replicons or PIV genomes. In H2A/C, 2A is a FMDV 2A protease gene and the vertical arrow shows the FMDV 2A-specific cleavage site. After co-electroporation of the *in vitro*-synthesized PIV and helper genomes, cells release only PIV genome-containing viral particles. The H1234/IRES/C helper is capable of replicating its own RNA. This latter helper construct is co-electroporated with the PIV genome into cells, and released virions contain both PIV and helper genomes. This two-component genome virus is passaged again and again on naïve cells. The helper construct provides an RNA packaging-competent capsid, and the PIV provides functional glycoproteins. However, both genomes need to be in the same cell. When the multiplicity of infection (MOI) is >1 (which occurs *in vitro*), infection is productive and spreading. When the MOI is < 1 (which occurs *in vivo*), helper and PIV genomes infect different cells and infection does not spread. No viremia develops, but replicating PIV produces genome-free virions.

Fig. 2. Alignment of the amino terminal sequence of VEEV capsid and its RK$^-$ mutant. Sequence involved in capsid dimerization during nucleocapsid formation encompasses amino acids 38-52. NLS sequence involved in its nuclear function and inhibition of cellular transcription encompasses amino acids 64-68. The latter mutations in NLS make capsid and virus noncytopathic and incapable of inhibiting the innate immune response.

Fig. 3. Schematic representation of VEEV PIV genome, its *in vitro* synthesis, packaging into infectious viral particles using either a capsid-producing cell line or helper RNA, and production of subviral particles. The latter particles are released by the cells infected by PIV.

5 **Figs. 4A-E.** (A) Schematic representation of wt, mutant virus and helper genomes. (B) Release of infectious virus particles from cells transfected with *in vitro*-synthesized viral RNAs (i.e., from DNA) and VEEV/RK-GFP+H/C helper RNAs. (C) Release of infectious virus particles from cells infected with VEEV/GFP or VEEV/RK-/GFP+H/C harvested after electroporation (MOI 10). (D) Analyses of viruses pelleted from the samples harvested either 10 after electroporation (EP) or after the next passage (aliquots correspond to 3 ml of harvested medium). (E) Analysis of density of viral particles released to the medium at passage 1, by ultracentrifugation in sucrose density gradient.

Fig. 5. Schematic representation of VEEV capsid-specific domains and other functional peptides. NES indicates nuclear export signal, NLS indicates nuclear import 15 signal and +++ indicates clusters of positively charged amino acids.

Fig. 6. Amino acid sequence of non-limiting exemplary capsid proteins of this invention.

DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, "a," "an" and "the" can mean one or more than one, depending on the context in which it is used. For example, "a" cell can mean one cell or multiple cells.

Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

25 Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of a compound or agent of this invention, dose, time, temperature, and the like, is meant to encompass variations of ± 20%, ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.

The term "alphavirus structural protein/protein(s)" refers to one or a combination of 30 the structural proteins encoded by alphaviruses. These are produced by the wild type virus as a polyprotein and are described generally in the literature as C-E3-E2-6k-E1. E3 and 6k serve as membrane translocation/transport signals for the two glycoproteins, E2 and E1.

Thus, use of the term E1 herein can refer to E1, 6k-E1, or E3-E2-6k-E1, and use of the term E2 herein can refer to E2, E3-E2, E2-6k, PE2, p62 or E3-E2-6k.

The terms "helper," "helper RNA" and "helper construct" are used interchangeably and refer to a nucleic acid molecule (either RNA or DNA) that encodes one or more alphavirus structural proteins. In the present invention, the helper construct generally encodes an RNA-binding competent alphavirus capsid protein. The capsid protein can comprise the amino acid sequence of what is known in the art to be the "wild type" capsid protein of a given alphavirus. Exemplary wild type amino acid sequences of various alphaviruses of this invention are provided herein below. The capsid protein encoded by a helper construct of this invention can also be an alphavirus capsid protein that has the function of binding and packaging alphavirus RNA and may have other modifications that distinguish its amino acid sequence from a wild type sequence, while retaining the RNA binding and packaging function. Optionally, the helper construct of this invention does not comprise a packaging signal. Optionally, the helper construct of this invention can comprise nucleotide sequence encoding all or a portion of one or more alphavirus nonstructural proteins or the helper construct of this invention does not comprise nucleotide sequence encoding all or a portion of one or more alphavirus nonstructural proteins. Further options for the helper construct of this invention can include a helper construct comprising nucleotide sequence encoding all or a portion of one or more alphavirus structural proteins (e.g., in addition to capsid) or the helper construct does not comprise nucleotide sequence encoding one or more alphavirus structural proteins (e.g., besides capsid).

The terms "helper cell" and "packaging cell" are used interchangeably herein and refer to a cell in which alphavirus PIV particles are produced. In particular embodiments, the helper cell or packaging cell of the present invention contains a stably integrated nucleotide sequence encoding an alphavirus RNA-binding competent capsid protein. The helper cell or packaging cell can be any cell that is alphavirus-permissive, i.e., that can produce alphavirus PIV particles upon introduction of a PIV genome. Alphavirus-permissive cells of this invention include, but are not limited to, Vero, baby hamster kidney (BHK), 293, 293T/17 (ATCC accession number CRL-11268), chicken embryo fibroblast (CEF), UMNSAH/DF-1 (ATCC accession number CRL-12203) and Chinese hamster ovary (CHO) cells.

An "isolated cell" as used herein is a cell or population of cells that have been removed from the environment in which the cell occurs naturally and/or altered or modified from the state in which the cell occurs in its natural environment. An isolated cell of this invention can be a cell, for example, in a cell culture. An isolated cell of this invention can

also be a cell that can be in an animal and/or introduced into an animal and wherein the cell has been altered or modified, e.g., by the introduction into the cell of an alphavirus PIV particle of this invention.

As used herein, an "alphavirus subgenomic promoter" or "26S promoter" is a promoter as originally defined in a wild type alphavirus genome that directs transcription of a subgenomic messenger RNA as part of the alphavirus replication process. Such a promoter can have a wild type sequence or a sequence that has been modified from wild type sequence but retains promoter activity.

The present invention is based on the unexpected discovery that pseudoinfectious alphavirus particles can infect cells and efficiently produce subviral particles (SVPs), lacking any viral genetic material, which serve as immunogens, without production of a spreading alphavirus infection.

Thus, in one embodiment, the present invention provides a pseudoinfectious alphavirus particle, comprising, consisting essentially of or consisting of, a nucleotide sequence encoding: a) alphavirus nonstructural proteins nsP1-4 (e.g., “wild type” nonstructural proteins or nonstructural proteins lacking mutations that alter the normal function of the nonstructural proteins), b) alphavirus structural proteins E2 and E1 (e.g., “wild type” structural proteins E2 and E1 or structural proteins E2 and E1 lacking mutations that alter the normal function of these structural proteins), and c) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, whereby binding of RNA to the capsid protein is substantially diminished or eliminated, thereby resulting in production of a noninfectious subviral particle lacking genetic material.

In certain embodiments, a nucleotide sequence of this invention, encoding: a) alphavirus nonstructural proteins nsP1-4 (e.g., “wild type” nonstructural proteins or nonstructural proteins lacking mutations that alter the normal function of the nonstructural proteins), b) alphavirus structural proteins E2 and E1 (e.g., “wild type” structural proteins E2 and E1 or structural proteins E2 and E1 lacking mutations that alter the normal function of these structural proteins), and c) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, whereby binding of RNA to the capsid protein is substantially diminished or eliminated can be in the form of RNA or DNA.

The present invention further provides a noninfectious subviral particle (SVP), comprising, consisting essentially of or consisting of, alphavirus structural proteins E2 and E1 and an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, and lacking genetic material.

The RNA binding domain in an alphavirus capsid protein comprises about the first 120 amino acids at the amino terminus of the protein. Thus, a pseudoinfectious alphavirus particle of this invention or a noninfectious subviral particle of this invention can comprise a capsid protein that is mutated at one or more positively charged amino acids (e.g., R, K, H, etc. as are well known in the art) within this RNA binding domain. By one or more is meant one, two, three, four, five, six, seven, eight, nine, ten, etc., as would be well understood by one of skill in the art.

For example, in one embodiment, the capsid protein can be mutated at one or more positively charged amino acids between amino acids 15 to 113 (with positively charged amino acids in this region shown in bold) of the amino acid sequence of the capsid protein of VEEV TC-83 strain: MFPFQPMYPM QPMPY**R**NPFA AP**RR**PWFP**R**T DPFLAMQVQE LTRSMANLTF KQ**RR**DAPPEG PSA**KK**P**KK**EA SQ**K**Q**K**GGGQG **KKKK**NQG**KKK** A**K**TGPPNP**K**A QNGN**KKK**TN**K** **K**PGKRQRMVM KLESDKTFPI MLEGKINGYA CVVGGKLFRP MHVEGKIDND VLAALKTKKA SKYDLEYADV PQNMRADTFK YTHEKPQGYY SWHHGAVQYE NGRFTVPKGV GAKGDSGRPI LDNQGRVVAI VLGGVNEGSR TALSVVMWNE KGVTVKYTPE NCEQW. Thus, a VEEV capsid protein of this invention can be mutated at R16, R23, R24, R29, R53, R54, K64, K65, K67, K68, K73, K75, K81, K82, K83, K84, K88, K89, K90, K92, K99, K105, K106, K107, K110, and/or K111, in any combination (numbering is according to VEEV TC83 capsid protein sequence provided herein).

Thus, the present invention provides a VEEV capsid protein that can be mutated at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 positively charged amino acids listed above in any combination, resulting in a capsid protein in which the positive charge in the RNA binding domain has been sufficiently altered to result in a capsid protein in which RNA binding is substantially diminished (e.g., at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or more reduction in RNA binding and RNA packaging activity relative to nonmutated capsid protein) or eliminated (100% reduction in binding activity relative to nonmutated capsid protein). Furthermore, these positively charged amino acids of the VEEV capsid protein are shown as mutation sites to produce a capsid protein that lacks RNA binding activity as only one example of this invention and it is to be understood that any one or more of the positively charged amino acids in the RNA binding domain of any other alphavirus (e.g., about the first 100, 110, 120, 125, 130, 135, 140 or 150 amino acids) can be mutated for the same purpose, i.e., to alter the positive charge in the RNA binding domain to result in an alphavirus capsid

protein in which RNA binding is substantially diminished or eliminated. These positively charged amino acids in alphaviruses other than VEEV will be numbered differently with respect to the amino acid sequence of a capsid protein of any given alphavirus, but one of skill in the art could readily identify which amino acids in the RNA binding domain are
5 positively charged and produce a capsid protein mutated at any of these amino acid sites in any combination and test the resulting mutated capsid protein for RNA binding activity, thereby producing and identifying a capsid protein of this invention. As nonlimiting examples, the amino acid sequence of the capsid protein of Chikungunya Thai strain, EEEV North America Florida 93 strain and WEEV McMillan strain are provided here as **Figure 6.**

In a particular embodiment, a VEEV capsid protein of this invention can comprise one or more of the following specific mutations in any combination (numbering is according to VEEV TC-83 capsid protein amino acid sequence provided herein): R16G, R23A, R24N, R29G, R53N, R54N, K64S, K65N, K67G, K68N, K73A, K75N, K81S, K82G, K83N, K84N, K88G, K89G, K90N, K92S, K99N, K105G, K106N, K107S, K110A, K111S (as shown in **Figure 2**). However, these particular mutations are examples of various amino acid substitutions that can be made for these positively charged amino acid residues in the RNA binding domain of an alphavirus capsid protein and it is to be understood that other amino acid substitutions can be made, in any combination, to reduce the positive charge of the RNA binding domain, resulting in an alphavirus capsid protein in which RNA binding activity has been diminished (e.g., substantially diminished) or eliminated. Nonlimiting examples of other amino acids that can be substituted for positively charged amino acids in the capsid protein of this invention include G, S, T, Q, N, V, A, D, E, L and I, in any combination.

10 The present invention further provides a population of alphavirus particles, comprising, consisting essentially of, or consisting of the pseudoinfectious alphavirus particles of this invention. Also provided herein is a population of subviral particles, comprising, consisting essentially of, or consisting of the noninfectious subviral particles of this invention. In some embodiments, the population of this invention contains no detectable
15 infectious particles as determined by passage on permissive cells in culture and in other embodiments, the population of this invention has no more than 1 infectious particle per 10^5 10^6, 10^7, 10^8, or 10^9 particles, as measured by passage on permissive cells in culture according to methods well known in the art.

Also provided in the present invention are compositions comprising the alphavirus
20 particles of this invention, such as a composition comprising each of the pseudoinfectious alphavirus particles, the noninfectious subviral particles and/or the populations of this

invention, in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject along with the selected particles, and/or populations thereof, without causing substantial deleterious biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. The pharmaceutically acceptable carrier is suitable for administration or delivery to humans and other subjects of this invention. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art (see, e.g., *Remington's Pharmaceutical Science*; latest edition). Pharmaceutical formulations, such as vaccines or other immunogenic compositions of the present invention can comprise an immunogenic amount of the PIV particles of this invention, in combination with a pharmaceutically acceptable carrier. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

The capsid protein of this invention can be the capsid protein of any alphavirus that can be employed in the production of the pseudoinfectious alphavirus particles of this invention. Thus, an alphavirus of this invention can be any alphavirus that can be employed in the production of the pseudoinfectious particles of this invention, as would be known to one of skill in the art and an alphavirus of this invention can be any alphavirus against which it is desirable or necessary to produce an immune response in a subject of this invention. Nonlimiting examples of an alphavirus of this invention include VEEV, WEEV, EEEV, Chikungunya virus, o'nyong-nyong virus, Ross River virus, Barmah Forest virus, Everglades, Mucambo, Pixuna, Semliki Forest virus, Middelburg, Getah, Bebaru, Mayaro, Una, Sindbis, Okelbo, Babanki, Fort Morgan, Ndumu and subgroups thereof as are known in the art. The complete genomic sequences, as well as the sequences of the various structural and non-structural proteins are available in the literature for numerous alphaviruses and include: Sindbis virus genomic sequence (GenBank® Database Accession Nos. J02363, NCBI Accession No. NC_001547), S.A.AR86 genomic sequence (GenBank Accession No. U38305), VEE genomic sequence (GenBank Accession No. L04653, NCBI Accession No. NC_001449), TC-83 vaccine strain of VEE (Kinney RM et al. (1989) *Virology* **170**:19-30; with correction noted in Kinney RM et al. (1993) *J. Virol.* **67**(3):1269-1277); Girdwood S.A genomic sequence (GenBank Accession No. U38304), Semliki Forest virus genomic sequence (GenBank Accession No. X04129, NCBI Accession No. NC_003215), and the TR339

genomic sequence (Klimstra et al. (1988) *J. Virol.* 72:7357; McKnight et al. (1996) *J. Virol.* 70:1981). These sequences and references are incorporated by reference herein.

A subject of this invention includes, but is not limited to, warm-blooded animals, e.g., humans, non-human primates, horses, cows, cats, dogs, pigs, rats, and mice.

5 The present invention also provides a method of eliciting or enhancing an immune response to an alphavirus in a subject, comprising administering to the subject an immunogenic amount of a pseudoinfectious alphavirus particle of this invention, a population of this invention and/or a pharmaceutical composition of this invention, thereby eliciting or enhancing an immune response to an alphavirus in the subject.

10 Also provided herein is a method of treating and/or preventing an alphavirus infection in a subject, comprising administering to the subject an immunogenic amount of a pseudoinfectious alphavirus particle of this invention, a population of this invention and/or a pharmaceutical composition of this invention, thereby treating and/or preventing an alphavirus infection in the subject.

15 As used herein, "eliciting an immune response," "enhancing an immune response" and "immunizing a subject" includes the development or enhancement, in a subject, of a humoral and/or a cellular immune response to an alphavirus protein of this invention (e.g., an immunogen, an antigen, an immunogenic peptide, and/or one or more epitopes). A "humoral" immune response, as this term is well known in the art, refers to an immune
20 response comprising antibodies, while a "cellular" immune response, as this term is well known in the art, refers to an immune response comprising T-lymphocytes and other white blood cells, especially the immunogen-specific response by HLA-restricted cytolytic T-cells, i.e., "CTLs."

An "immunogenic amount" is an amount of the alphavirus PIV particles in the
25 populations of this invention that is sufficient to elicit or enhance an immune response in a subject to which the population of particles is administered or delivered. An amount of from about 10^4 to about 10^9, especially 10^6 to 10^8, infectious units, or "IU," as determined by assays well known in the art, per dose is considered suitable, depending upon the age and species of the subject being treated. Administration may be by any suitable means, such as
30 intraperitoneally, intramuscularly, intranasally, intravenously, intradermally (e.g., by a gene gun), intrarectally and/or subcutaneously. The compositions herein may be administered via a skin scarification method, and/or transdermally via a patch or liquid. The compositions can be delivered subdermally in the form of a biodegradable material that releases the compositions over a period of time.

As used herein, "effective amount" refers to an amount of a population or composition or formulation of this invention that is sufficient to produce a desired effect, which can be a therapeutic effect. The effective amount will vary with the age, general condition of the subject, the severity of the condition being treated, the particular agent administered, the duration of the treatment, the nature of any concurrent treatment, the pharmaceutically acceptable carrier used, and like factors within the knowledge and expertise of those skilled in the art. As appropriate, an "effective amount" in any individual case can be determined by one of ordinary skill in the art by reference to the pertinent texts and literature and/or by using routine experimentation. (*See*, for example, Remington, *The Science And Practice of Pharmacy* (20th ed. 2000)).

Alternatively, pharmaceutical formulations of the present invention may be suitable for administration to the mucous membranes of a subject (e.g., via intranasal administration, buccal administration and/or inhalation). The formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well known in the art.

Also, the composition of this invention may be used to infect or be transfected into dendritic cells, which are isolated or grown from a subject's cells, according to methods well known in the art, or onto bulk peripheral blood mononuclear cells (PBMC) or various cell subfractions thereof from a subject.

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art while the compositions of this invention are introduced into the cells or tissues.

Immunogenic compositions comprising a population of the particles of the present invention may be formulated by any means known in the art. Such compositions, especially vaccines, are typically prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. Lyophilized preparations are also suitable.

The active immunogenic ingredients (e.g., the PIV particles) are often mixed with excipients and/or carriers that are pharmaceutically acceptable and/or compatible with the active ingredient. Suitable excipients include but are not limited to sterile water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof, as well as stabilizers, e.g., HSA or other suitable proteins and reducing sugars.

In addition, if desired, the vaccines or immunogenic compositions may contain minor amounts of auxiliary substances such as wetting and/or emulsifying agents, pH buffering agents, and/or adjuvants that enhance the effectiveness of the vaccine or immunogenic

composition. Examples of adjuvants which may be effective include but are not limited to: QS-21, Freund's adjuvant (complete and incomplete), aluminum salts (alum), aluminum phosphate, aluminum hydroxide; N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP); N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP); N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE); and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

Additional examples of adjuvants can include, but are not limited to, immunostimulating agents, such as bacterial cell wall components or synthetic molecules, or oligonucleotides (e.g., CpGs) and nucleic acid polymers (both double stranded and single stranded RNA and DNA), which can incorporate alternative backbone moieties, e.g., polyvinyl polymers.

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies or cytotoxic T-cells directed against the immunogenic product of the alphavirus PIV particles resulting from administration of the particle-containing composition in a vaccine formulation that also comprises an adjuvant or combination of adjuvants. Such additional formulations and modes of administration as are known in the art may also be used.

Adjuvants can be combined, either with the compositions of this invention or with other vaccine formulations that can be used in combination with the compositions of this invention.

The compositions of the present invention can also include other medicinal agents, pharmaceutical agents, carriers, and diluents.

The compositions of this invention can be optimized and combined with other vaccination regimens to provide the broadest (i.e., covering all aspects of the immune response, including those features described hereinabove) cellular and humoral responses possible. In certain embodiments, this can include the use of heterologous prime-boost strategies, in which the compositions of this invention are used in combination with a composition comprising one or more of the following: immunogens derived from a pathogen or tumor, recombinant immunogens, naked nucleic acids, nucleic acids formulated with lipid-containing moieties, non-alphavirus vectors (including but not limited to pox vectors, adenoviral vectors, adeno-associated viral vectors, herpes virus vectors, vesicular stomatitis

virus vectors, paramyxoviral vectors, parvovirus vectors, papovavirus vectors, retroviral vectors, lentivirus vectors), and other alphavirus vectors.

The immunogenic (or otherwise biologically active) alphavirus particle-containing populations and compositions of this invention are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which can generally ne in the range of about 10^4 to about 10^{10} infectious units in a dose (e.g., about 10^4, about 10^5, about 10^6, about 10^7, about 10^8, about 10^9, or about 10^{10}), depends on the subject to be treated, the route by which the particles are administered or delivered, the immunogenicity of the expression product, the types of effector immune responses desired, and the degree of protection desired. In some embodiments, doses of about 10^6, about 10^7, and about 10^8 infectious units may be particularly effective in human subjects. Effective amounts of the active ingredient required to be administered or delivered may depend on the judgment of the physician, veterinarian or other health practitioner and may be specific for a given subject, but such a determination is within the skill of such a practitioner.

The compositions and formulations of this invention may be given in a single dose or multiple dose schedule. A multiple dose schedule is one in which a primary course of administration may include 1 to 10 or more separate doses, followed by other doses administered at subsequent time intervals as required to maintain and or reinforce the desired effect (e.g., an immune response), e.g., weekly or at 1 to 4 months for a second dose, and if needed, a subsequent dose(s) after several months (e.g., 4 or 6 months)/years.

Efficacy of the treatment methods of this invention can be determined according to well known protocols for determining the outcome of a treatment of a disease or infection of this invention. Determinants of efficacy of treatment, include, but are not limited to, overall survival, disease-free survival, improvement in symptoms, time to progression and/or quality of life, etc., as are well known in the art.

"Treat" or "treating" or "treatment" refers to any type of action that imparts a modulating effect, which, for example, can be a beneficial effect, to a subject afflicted with a disorder, disease or illness, including improvement in the condition of the subject (*e.g.,* in one or more symptoms), delay or reduction in the progression of the condition, delay of the onset of the disorder , disease or illness, and/or change in any of the clinical parameters of a disorder, disease or illness, etc., as would be well known in the art.

The terms "preventing" or "prevent" as used herein refers to the prophylactic administration of the alphavirus PIV particles of this invention to a subject to protect the

subject from becoming infected by the alphavirus and/or to reduce the severity of an alphavirus infection in a subject who becomes infected. Such as subject can be a healthy subject for whom prevention of infection by an alphavirus is desirable. The subject can also be at increased risk of becoming infected by an alphavirus and therefore desires and/or is in need of the methods of preventing alphavirus infection provided herein.

Also provided in this invention are methods of producing the pseudoinfectious alphaviruses of this invention, for use in the therapeutic methods described above. These methods of production generally involve the introduction of the PIV genome (with a mutated capsid protein) into a cell in which an RNA-binding competent alphavirus capsid protein is also present and thus available in *trans* to produce the PIV particles of this invention. The RNA-binding competent capsid protein can be produced as the result of expression of a nucleotide sequence encoding the capsid protein, wherein the nucleotide sequence has been stably integrated into the genome of the cell. In this embodiment, upon introduction of the PIV genome into the cell in which capsid protein is being produced, the structural proteins E2 and E1 are provided, allowing for assembly of the PIV particles that are capable of a single round of infection. In other embodiments, the PIV genome can be introduced into the cell along with a helper construct that encodes an RNA-binding competent alphavirus capsid protein. Thus, the capsid protein is available in *trans* from the helper construct for assembly of the PIV particles. Numerous helper constructs, both RNA and DNA, are known in the art. Some nonlimiting examples of helper constructs of this invention are described in **Figure 1**. Schematics of the steps of producing PIV particles according to these respective embodiments are shown in **Figure 3.**

Thus, in one embodiment, the present invention provides a method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing into an alphavirus permissive cell: a) a nucleic acid molecule encoding: i) alphavirus nonstructural proteins nsP1-4, ii) alphavirus structural proteins E2 and E1, and iii) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain; and b) a helper nucleic acid molecule encoding alphavirus capsid protein, whereby the structural proteins E2, E1 and capsid are produced in the cell; and maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

In a further embodiment, the present invention provides a method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing a nucleic acid molecule encoding: i) alphavirus nonstructural proteins nsP1-4, ii) alphavirus structural proteins E2 and E1, and iii) an alphavirus capsid protein mutated at one or more positively

charged amino acids in the RNA binding domain, into a cell containing a nucleic acid molecule encoding alphavirus capsid protein, whereby the structural proteins E2, E1 and capsid are produced in the cell; and maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

The present invention further provides a population of alphavirus particles comprising the pseudoinfectious alphavirus particles produced by the methods described herein.

5 It is understood that the foregoing detailed description is given merely by way of illustration and that modifications and variations may be made therein without departing from the spirit and scope of the invention.

EXAMPLES

10 The *Alphavirus* genus in the Togaviridae family includes a number of important human and animal pathogens (26). The New World alphaviruses, which include Venezuelan (VEEV), eastern (EEEV) and western equine encephalitis (WEEV) viruses, represent a serious public health threat in the US (53, 68-70). They continuously circulate in the Central, South and North Americas, including the US, and cause periodic, extensive equine epizootics 15 and epidemics of encephalitis among humans with frequent lethal outcomes and neurological sequelae.

The overall mortality rates are <1% (67), 30–80% (66) and 1-5% (52) for human cases of VEE, EEE and WEE, respectively. However, these values can increase following aerosol infection (51). WEEV, EEEV and particularly VEEV have a potential for use by 20 terrorists and as biological warfare agents. They are very "user friendly" compared to many other viral agents. These viruses can replicate to very high titers, approaching 10^{10} infectious units/ml, in a wide variety of cell types, remain highly stable in a lyophilized form and are highly infectious by aerosol.

In spite of the continuous public threat, no effective antivirals and/or vaccines have 25 been developed against VEEV, EEEV or WEEV infections (4). There exists an attenuated VEEV TC-83 vaccine strain, developed more than four decades ago by serial passaging of the virulent TrD strain in cell culture (2). TC-83 is currently the only VEEV strain available for vaccination of laboratory workers and military personnel. However, in nearly 40% of people, vaccination results in a disease with the symptoms typical of natural VEEV infection 30 (4). The attenuated phenotype of TC-83 relies on two point mutations, and therefore a strong probability of reversion to a more virulent phenotype during replication *in vivo* remains a high risk (33). Moreover, VEEV TC-83 can be transmitted by mosquito vectors, making the

possibility of reversion even more likely (47). A formalin-inactivated version of the TC-83 vaccine, C-84 (50), requires repeated boosters because the level of induced neutralizing antibodies is low and they persist for only a short time.

Attenuation of the VEEV TrD strain was also achieved by introduction of lethal mutations into the E3/E2 furin cleavage site of an infectious cDNA clone, followed by selection of a second-site suppressor mutation in the E1 glycoprotein (12). This virus is attenuated (63), but clinical trials with this strain have been stopped due to high rates of adverse reactions in volunteers.

The experimental DNA and adenovirus vaccine candidates demonstrate numerous drawbacks, including very low efficiencies in the induction of neutralizing Ab and pre-existing vector immunity. The virus-like particle (VLP)-based vaccines might be promising in terms of safety, but are very expensive and induce only a short-lived Ab response (3).

Therefore, a need for the development of safe and efficient vaccines against these highly pathogenic New and Old World alphaviruses remains a priority. This need became even more evident recently, when the number of confirmed and lethal cases of EEE among humans and horses in Florida, South Carolina, Georgia, Mississippi, Alabama, Michigan and other states dramatically increased.

The invention described herein is based on three important discoveries provided in the studies described herein: i) identification of a conserved sequence in the alphavirus nsP1-coding fragment, which based on these data, appears to function as a universal packaging signal in many alphavirus serogroups during virion assembly; ii) demonstration of the ability of capsid protein containing mutations at the positively charged amino acid sites to very efficiently form virions lacking viral genetic material; and iii) identification of a capsid-specific peptide that functions in the inhibition of nucleocytoplasmic trafficking, induction of transcriptional shutoff and cytopathic effect (CPE) development in the infected cells.

The present invention is thus directed to designing live alphavirus variants possessing a highly attenuated phenotype, but producing virus-specific antigens for induction of immunity efficiently (e.g., in some embodiments, as efficiently as a wild type (wt) virus). The present invention is also directed to the development of variants that are reduced in the ability to induce viremia and in some embodiments do not induce viremia at all. The modifications will not affect viral RNA replication, structural protein production or the ability to produce viral (or virus-like) particles. However, the released *in vivo* virions will contain no or very low levels of viral genetic material, and as a result, in particular embodiments, will develop no detectable spreading infection *in vivo*. Thus, the new variants

combine the best characteristics of live attenuated vaccine candidates, which are induction of balanced, efficient and long-term immune response and easy propagation in cultured cells, while also matching inactivated or VLP vaccines, in terms of their safety.

The effectiveness of vaccine candidates is largely determined by the availability of a
5 system for their large-scale production in order to be useful to the public. Therefore, a further aspect of this invention is an *in vitro trans*-complementation-based packaging system for the large-scale packaging of defective viral genomes into infectious viral particles, which facilitates *in vivo* applications.

Successful *trans*-complementation systems for packaging of defective flavivirus
10 genomes have been developed (Mason et al. "Production and characterization of vaccines based on flaviviruses defective in replication" *Virology* 351(2):432-43 (2006); Shustov et al. "Efficient, trans-complementing packaging systems for chimeric, pseudoinfectious dengue 2/yellow fever viruses" *Virology* 400(1):8-17 (2010)). These defective flaviviruses are now in pre-clinical trials as vaccine candidates for TBE and JEV (under the trade name of
15 RepliVax).

Although the studies described herein are focused on VEEV, it is to be understood that the mechanisms described herein for making and using VEEV PIV particles are common to all of the alphaviruses. Therefore, the results can be applied to all of the New and Old World alphaviruses, such as, e.g., EEEV and WEEV, CHIKV and o'nyong-nyong virus,
20 among others. These studies provide a balanced combination of basic, mechanistic research of alphavirus genome packaging and particle formation, and application of the results for the development of a fundamentally new type of safe and efficient alphavirus vaccine.

The present invention is directed to the design and development of live, attenuated encephalitogenic RNA^+ viruses, which combine the safety of inactivated virus and efficiency
25 of live vaccines. The key elements of this strategy are as follows: A) High safety of the new variants results from the poor efficiency of packaging of viral genome RNA into viral particles during replication *in vivo*. In some embodiments, the variant will be incapable or almost completely incapable of packaging viral genomes. B) High efficiency of newly designed alphaviruses in the induction of an immune response is based, in part, on eliciting
30 balanced cellular and neutralizing antibody responses. During *in vivo* replication, these viruses demonstrate high levels of RNA replication and structural protein production. The latter proteins are presented at the plasma membrane and released in the form of immunogenic, virus-like particles (e.g., subviral particles (SVPs)), containing no viral genetic material. However, these viruses induce only a low level viremia or no viremia at all in terms

of infectious virus circulation. C) In some embodimens, the attenuated, defective in viral genome packaging phenotype will be irreversible. D) Application of the attenuated viruses as vaccines includes the development of an *in vitro* system for the large-scale packaging of these mutated genomes into infectious viral particles for their delivery into cells *in vivo*.

5 By introducing multiple, rationally designed mutations into alphavirus capsid protein or into newly identified alphavirus universal RNA packaging signal, alphaviruses will be developed that retain high levels of RNA replication, and production of structural proteins which are released in the form of non-infectious, genome-deficient virus-like particles. IN representative embodiments, these viruses are capable of inducing a balanced combination of
10 cellular immune response and high levels of neutralizing Abs.

Development and analysis of pseudoinfectious (PIV) alphaviruses.

Experiments described herein are directed to the development of prototype, defective, pseudoinfectious alphaviruses (PIV), which can be propagated in tissue culture, but are able to release only genome-free, immunogenic virions in vivo. These studies are directed to the
15 development of a VEEV capsid protein that is incapable of packaging viral genomes but retains the ability to efficiently form nucleocapsids and genome-free viral particles.

Capsid protein structure. VEEV capsid protein is composed of 275 amino acids (Kinney et al. "Nucleotide sequences of the 26S mRNAs of the viruses defining the Venezuelan equine encephalitis antigenic complex" *Am J Trop Med Hyg.* 59(6):952-64
20 (1998)) and contains a number of sequences playing various roles in virus replication (**Fig. 5**). It contains two large structural domains (29): i) The highly ordered carboxy terminal domain is located between amino acids 126 and 275 of the VEEV capsid protein. It functions as a self-protease in processing of the structural polyprotein and is required for co- and post-translational cleavage of the capsid protein from p62 (the precursor of E2 glycoprotein). The
25 carboxy terminal domain has also been proposed as a major determinant for assembly of the capsid proteins into icosahedral nucleocapsids. ii) The amino terminal domain (aa 1-126), in contrast to the protease domain, has no defined secondary structure and is highly positively charged. This domain is responsible for binding to viral genomic RNA and packaging it into infectious virions. However, it also has a number of other important functions. It possesses a
30 small alpha helical peptide, Helix I, located between aa 34-52 that functions as a capsid dimerization signal in nucleocapsid assembly (48, 49, 59). The same helix is also a supraphysiological nuclear export signal (supraNES) playing a role in VEEV capsid-specific inhibition of nuclear trafficking (5). Another short sequence (aa 64-68) in VEEV capsid protein is a nuclear export signal (NLS) (5, 6). Supra NES and NLS bind exportin-α/β and

importin CRM1 to form a tetrameric complex that blocks the NPC function and nuclear import. This ultimately leads to inhibition of cellular transcription and downregulation of the innate immune response (5, 6). The third important amino acid sequence is a highly conserved peptide (CP) (aa 111-126 in VEEV capsid protein) that has been shown to play a role in the packaging of SINV genomes (65). Mutations in the latter peptide have a strong negative effect on virus titers (measured in infectious units) and packaging of virus-specific RNA.

***In vitro* studies.** In these studies a VEEV capsid protein was designed that is incapable of packaging viral genomes. To achieve this, an extensive mutagenesis of the amino terminal fragment of the VEEV-derived capsid gene was performed and almost all of the arginines and lysines within the 1-110 aa region were mutated to glycine, alanine and/or asparagine. Based on a computer prediction, the introduced 26 mutations did not change the disordered secondary structure of this protein fragment, but dramatically reduced the positive charge of the amino terminal domain of the protein (e.g., by leaving only 7 of 33 positively charged amino acids remaining) and were expected to have a strong negative effect on the capsid protein's ability to bind and package ssRNAs. The only peptides that remained unmodified were the above-described Helix I, required for capsid dimerization and nucleocapsid formation, and the CP peptide.

The designed recombinant viral genome (VEEV/RK$^-$/GFP) was synthesized *in vitro* and transfected into BHK-21 cells by electroporation either alone or in the presence of capsid-encoding helper RNA (**Figs. 4A-B**). An equal amount of the wt VEEV/GFP RNA was transfected as a positive control. The recombinant capsid mutant did not cause any cytopathic effect (CPE) and released infectious virus to a very low concentration (~10^4 infectious units/ml), compared to 10^{10} plaque forming units/ml (PFU/ml) of the control VEEV/GFP (**Fig. 4B**). Thus, each electroporated cell, containing VEEV/GFP genome released ~10,000 infectious viral particles, but only one of 10 cells containing the replicating capsid mutant genome released one infectious virion. This variant was unable to produce plaques, and its titers were determined only based on green fluorescent protein (GFP) fluorescence of the infected cells. Stocks could not be prepared with a concentration above 10^5 infectious units/ml. Accordingly, at the next passage, due to very low efficiency of infectious virus release, the capsid mutant was unable to develop a productive, spreading infection in tissue culture. However, the inefficient genome packaging by VEEV/RK$^-$/GFP capsid could be complemented by supplying the RNA packaging-competent capsid in *trans*. Co-electroporation of VEEV/RK$^-$/GFP and capsid-encoding helper RNA (**Fig. 4A**) caused a

dramatic increase in infectious titers (**Fig. 4B**), and the released particles contained both wt and mutated capsid protein (Fig. 4D, line 1). Helper RNA was packaged very inefficiently, and at the next passage, cells released VEEV/RK⁻/GFP genome-containing, infectious virions to very low levels (**Fig. 4C**). However, these cells produced the genome-free viral particles with great efficiency, and the harvested samples contained as many virions as the samples harvested from the VEEV/GFP-infected cells (**Fig. 4D**, compare lines 2 and 3, which represent viral particles pelleted from 3 ml of media). These infectious virions containing the VEEV/RK⁻/GFP genome are termed "pseudoinfectious virus (PIV)." Particles released from VEEV/RK⁻/GFP-infected cells demonstrated lower density (**Fig. 4E**), additionally indicating lack of packaged RNA, which correlated with very low infectivity of released virions.

Testing of residual pathogenicity and immunogenicity of the recombinant viruses.

Experimental design. To test neurovirulence of the designed, recombinant viruses, 6-days-old NIH-Swiss mice are infected intracerebrally (i.c.) with 20 μl (10^6 infectious units) of packaged VEEV/RK⁻ or control VEEV TC-83. At this dose, TC-83, the experimental vaccine, is universally lethal for these mice and, thus, represents a good control for comparative studies. Eight pups are used for each virus, and they are observed for 12 days for any signs of disease, which include development of paralysis and weight loss. If mice develop severe paralysis, they are euthanized. In this scenario, virus titers in their brain tissues are analyzed. In parallel, each day post infection, 3 mice from each group will be euthanized and virus titers in the brains will be assessed for 5 days to evaluate the levels of their replication even if the variants are incapable of causing death.

Testing the induction of neutralizing antibodies and cellular immune response.

Experimental design. Groups of 6-week-old NIH Swiss mice (8 mice per group) are infected subcutaneously (s.c.) with 10^5 and 10^7 infectious units of packaged VEEV/RK⁻ or control VEEV TC-83. At days 7, 14 and 28 post-infection, blood samples are collected and tested for levels of neutralizing antibodies (Ab) using the $PRNT_{80}$ assay (Wang et al. "Chimeric alphavirus vaccine candidates for chikungunya" *Vaccine* 26(39):5030-9 (2008)), with VEEV TC-83 virus.

In order to assess whether the vaccine constructs elicit cellular immune responses, the total and virus-specific CD4 and CD8 T cell populations are analyzed using a series of established flow cytometry-based assays. The basic approach encompasses infecting separate cohorts of 6-week-old BALB/c mice (6 mice per group) with 10^6 infectious units of VEEV/mutPS/CmutNLS, packaged VEEV/RK⁻ or control VEEV TC-83. To assess the induction and longevity of the virus-specific T cell responses, PBMC, spleens and lymph

nodes are harvested at days 8 (effector phase), 30 (early memory phase) and 60 (memory phase) following infection. First, the global activation of CD4 and CD8 T cells following infection are evaluated by performing polychromatic flow cytometry. Second, the frequencies, phenotypes and functions of the VEEV-specific responses are measured
5 following stimulation of the cell populations infected with tested noncytopathic viruses or control, uninfected BALB/c cl.7 and A20 cells. The co-culture with the virus-infected stimulators activates any VEEV-specific cells present. This activation is determined by intracellular staining for panels of effector molecules including IFN-g, TNF-a, and IL-2. By using conventional experimental approaches, an assessment can be made of the establishment
10 of effector and memory T cells, their functional quality and whether the candidates induce distinct subsets of memory T cells.

Additional information regarding the protective efficacy of the viruses used is generated in the *in vivo* CTL assays (21). Taken together, these data provide information about the cellular component of the immune response induced by designed viral mutants.
15 The most favorable result is if a single immunization is sufficient for induction of neutralizing Ab functioning at 1:80 or higher dilutions and induction of cellular immune response at least comparable to that induced by VEEV TC-83. However, if this is not the case, the immune response is boosted with a second immunization 28 days after the first immunization and the assessment of the Ab titers using the $PRNT_{80}$ test and other assays is
20 repeated.

Efficacy studies in an animal model.

Experimental design. Constructs selected in the immunological studies are further evaluated in challenge experiments. Groups of 6-week-old NIH Swiss mice (12 mice per group, 2 viruses, 2 doses and a control group) are immunized s.c. with 10^5 and 10^6 infectious
25 units of packaged VEEV/RK^- PIV. At day 28 post immunization, these mice are infected with 1,000 LD_{50} of VEEV TRD by s.c. route. The same doses are used for challenging sham-immunized mice. Mice are evaluated daily for 12 days for any signs of disease, which include development of paralysis and weight loss. If mice develop severe paralysis, they are euthanized. The same mice are used for the analysis of viremia development. Blood samples
30 are collected by retro-orbital puncture at days 1, 2, 3 and 4 post infection of immunized and unimmunized mice.

As will be understood by one skilled in the art, there are several embodiments and elements for each aspect of the claimed invention, and all combinations of different elements are incorporated herein as embodiments of this invention, so the specific combinations

exemplified herein are not to be construed as limitations in the scope of the invention as claimed. If specific elements are removed or added to the group of elements available in a combination, then the group of elements is to be construed as having incorporated such a change.

5 All references cited herein, including non-patent publications, patent applications, GenBank® Database accession numbers and patents, are incorporated by reference herein in their entireties to the same extent as if each was individually and specifically indicated to be incorporated by reference, and was reproduced in its entirety herein.

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What is claimed is:

1. A pseudoinfectious alphavirus particle comprising a genome encoding:

a) alphavirus nonstructural proteins nsP1-4,

b) alphavirus structural proteins E2 and E1, and

c) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, whereby binding of RNA to the capsid protein is substantially diminished or eliminated, thereby resulting in production of a noninfectious subviral particle lacking genetic material.

2. A noninfectious subviral particle, comprising alphavirus structural proteins E2 and E1 and an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain, and lacking genetic material.

3. The pseudoinfectious alphavirus particle of claim 1 or the noninfectious subviral particle of claim 2, wherein the capsid protein is mutated at one or more positively charged amino acids between amino acids 16 to 112 of the amino acid sequence:
MFPFQPMYPMQPMPYRNPFAAPRRPWFPRTDPFLAMQVQELTRSMANLTF
KQRRDAPPEGPSAKKPKKEASQKQKGGGQGKKKKNQGKKKAKTGPPNPKA
QNGNKKKTNKKPGKRQRMVMKLESDKTFPIMLEGKINGYACVVGGKLFRP
MHVEGKIDNDVLAALKTKKASKYDLEYADVPQNMRADTFKYTHEKPQGYY
SWHHGAVQYENGRFTVPKGVGAKGDSGRPILDNQGRVVAIVLGGVNEGSR
TALSVVMWNEKGVTVKYTPENCEQW.

4. The pseudoinfectious alphavirus particle of claim 1 or the noninfectious subviral particle of claim 2, wherein the capsid protein is mutated at one or more amino acids of the amino acid sequence:
MFPFQPMYPMQPMPYRNPFAAPRRPWFPRTDPFLAMQVQELTRSMANLTF
KQRRDAPPEGPSAKKPKKEASQKQKGGGQGKKKKNQGKKKAKTGPPNPKA
QNGNKKKTNKKPGKRQRMVMKLESDKTFPIMLEGKINGYACVVGGKLFRP
MHVEGKIDNDVLAALKTKKASKYDLEYADVPQNMRADTFKYTHEKPQGYY
SWHHGAVQYENGRFTVPKGVGAKGDSGRPILDNQGRVVAIVLGGVNEGSR
TALSVVMWNEKGVTVKYTPENCEQW, selected from the group consisting of:

1) R16;
2) R23;
3) R24;
4) R29;
5) R53;
6) R54;
7) K64;
8) K65;
9) K67;
10) K68;
11) K73;
12) K75;
13) K81;
14) K82;
15) K83;
16) K84;
17) K88;
18) K89;
19) K90;
20) K92;
21) K99;
22) K105;
23) K106;
24) K107;
25) K110;
26) K111; and
27) any combination of (1) through (26) above.

5. The pseudoinfectious alphavirus particle of claim 1 or the noninfectious subviral particle of claim 2, wherein the capsid protein comprises a mutation selected from the group consisting of:

1) R16G;
2) R23A;
3) R24N;

4) R29G;

5) R53N;

6) R54N;

7) K64S;

8) K65N;

9) K67G;

10) K68N;

11) K73A;

12) K75N;

13) K81S;

14) K82G;

15) K83N;

16) K84N;

17) K88G;

18) K89G;

19) K90N;

20) K92S;

21) K99N;

22) K105G;

23) K106N;

24) K107S;

25) K110A;

26) K111S; and

27) any combination of (1) through (26) above.

6. A population of alphavirus particles, comprising the pseudoinfectious alphavirus particles of claim 1.

7. A population of subviral particles, comprising the noninfectious subviral particles
5 of claim 2.

8. The pseudoinfectious alphavirus particle of claims 1, 3, 4 or 5, the subviral particle of claims 2, 3, 4 or 5, or the population of claims 6 or 7, wherein the capsid protein is from an

alphavirus selected from the group consisting of VEEV, WEEV, EEEV, Chikungunya virus, o'nyong-nyong virus, Ross River virus and Barmah Forest virus.

9. A pharmaceutical composition comprising the pseudoinfectious alphavirus particle of claims 1, 3, 4 or 5, the subviral particle of claims 2, 3, 4 or 5, or the population of claims 6 or 7 in a pharmaceutically acceptable carrier.

10. A method of eliciting or enhancing an immune response to an alphavirus in a subject, comprising administering to the subject an immunogenic amount of the pseudoinfectious alphavirus particle of claims 1, 3, 4 or 5, the population of claim 6 and/or the pharmaceutical composition of claim 9, thereby eliciting or enhancing an immune response to an alphavirus in the subject.

11. A method of treating and/or preventing an alphavirus infection in a subject, comprising administering to the subject an immunogenic amount of the pseudoinfectious alphavirus particle of claims 1, 3, 4, or 5, the population of claim 6 and/or the pharmaceutical composition of claim 9, thereby treating and/or preventing an alphavirus infection in the subject.

12. A method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing into a cell:

a) a nucleic acid molecule encoding:

i) alphavirus nonstructural proteins nsP1-4,

ii) alphavirus structural proteins E2 and E1, and

iii) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain; and

b) a helper nucleic acid molecule encoding alphavirus RNA-binding competent capsid protein; and

maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

13. A method of producing pseudoinfectious alphavirus particles in cell culture, comprising introducing a nucleic acid molecule encoding:

i) alphavirus nonstructural proteins nsP1-4,

ii) alphavirus structural proteins E2 and E1, and

iii) an alphavirus capsid protein mutated at one or more positively charged amino acids in the RNA binding domain,

into a cell containing a nucleic acid molecule encoding an alphavirus RNA-binding competent capsid protein; and

maintaining the cell in culture to produce the pseudoinfectious alphavirus particles.

14. A population of alphavirus particles comprising the pseudoinfectious alphavirus particles produced by the method of claim 12 or claim 13.

Fig. 1

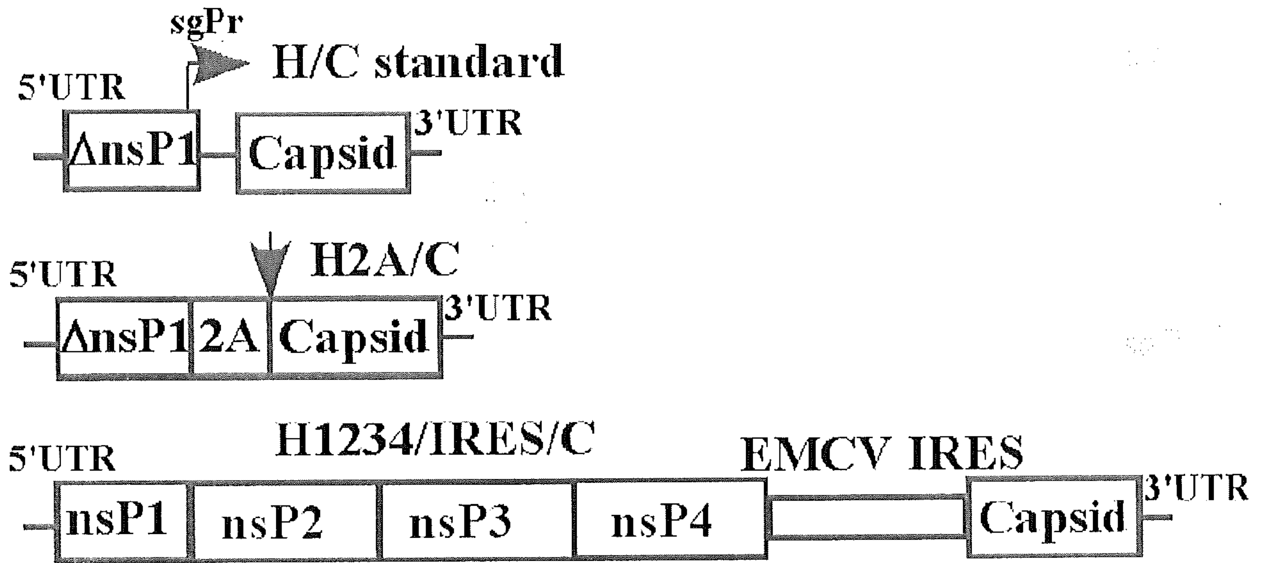

Fig. 2

```
1                                      40
MFPFQPMYPMQPMPYRNPFAAPRRPWFPRTDPFLAMQVQE
---------------G------AN----G-----------

41                                     80
LTRSMANLTFKQRRDAPPEGPSAKKPKKEASQKQKGGGQG
------------NN---------SN-GN----A-N-----

81                            conserved 120
KKKKNQGKKKAKTGPPNPKAQNGNKKKTNKKPGKRQRMVM
SGNNN--GGN-S------N-----GNS--AS---------
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Fig. 3

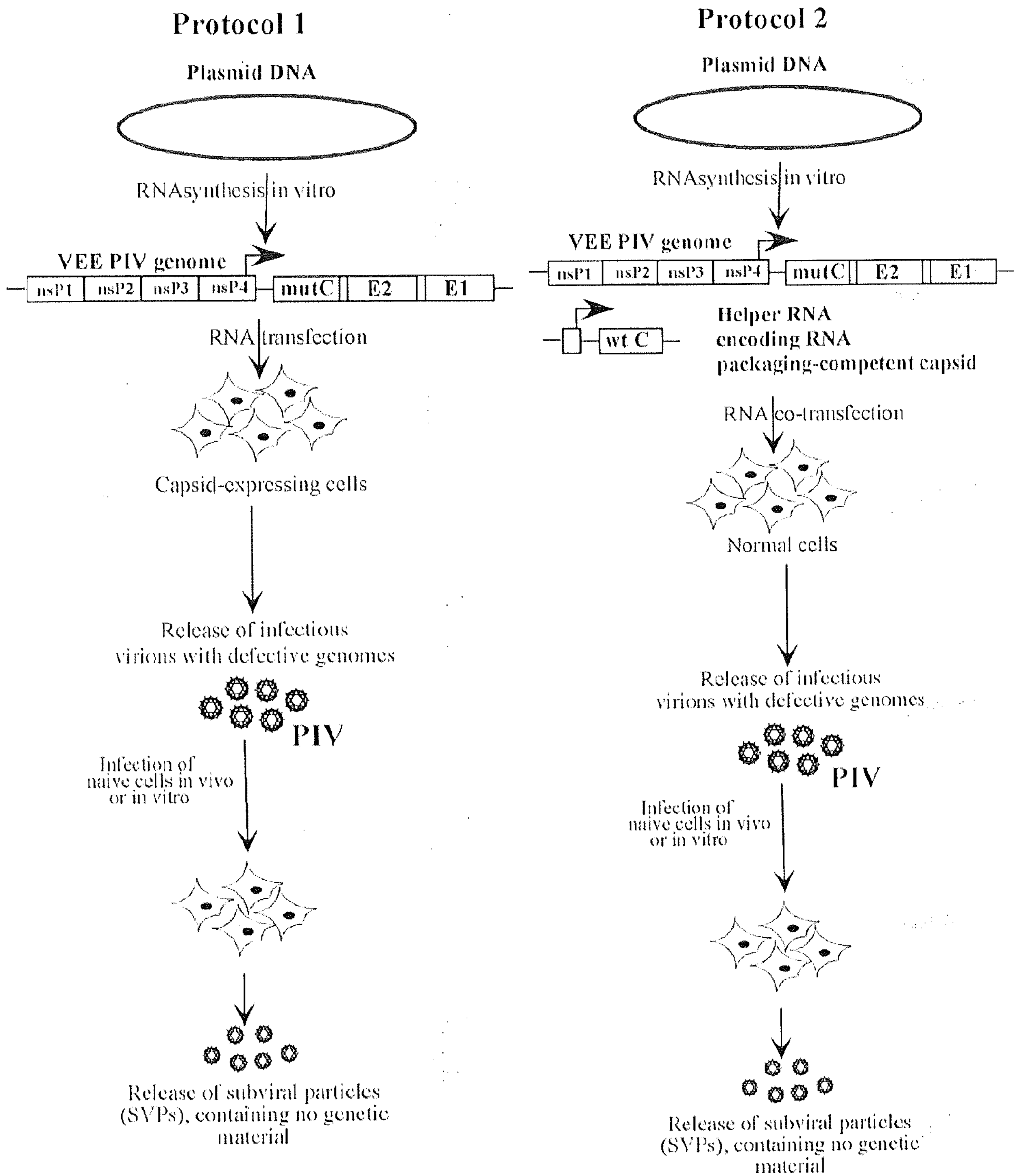
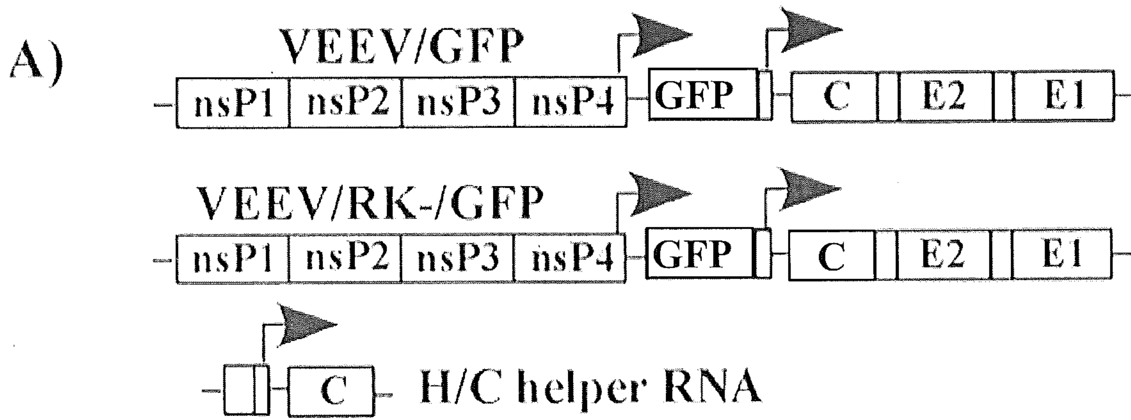


Fig. 4

A)



B)

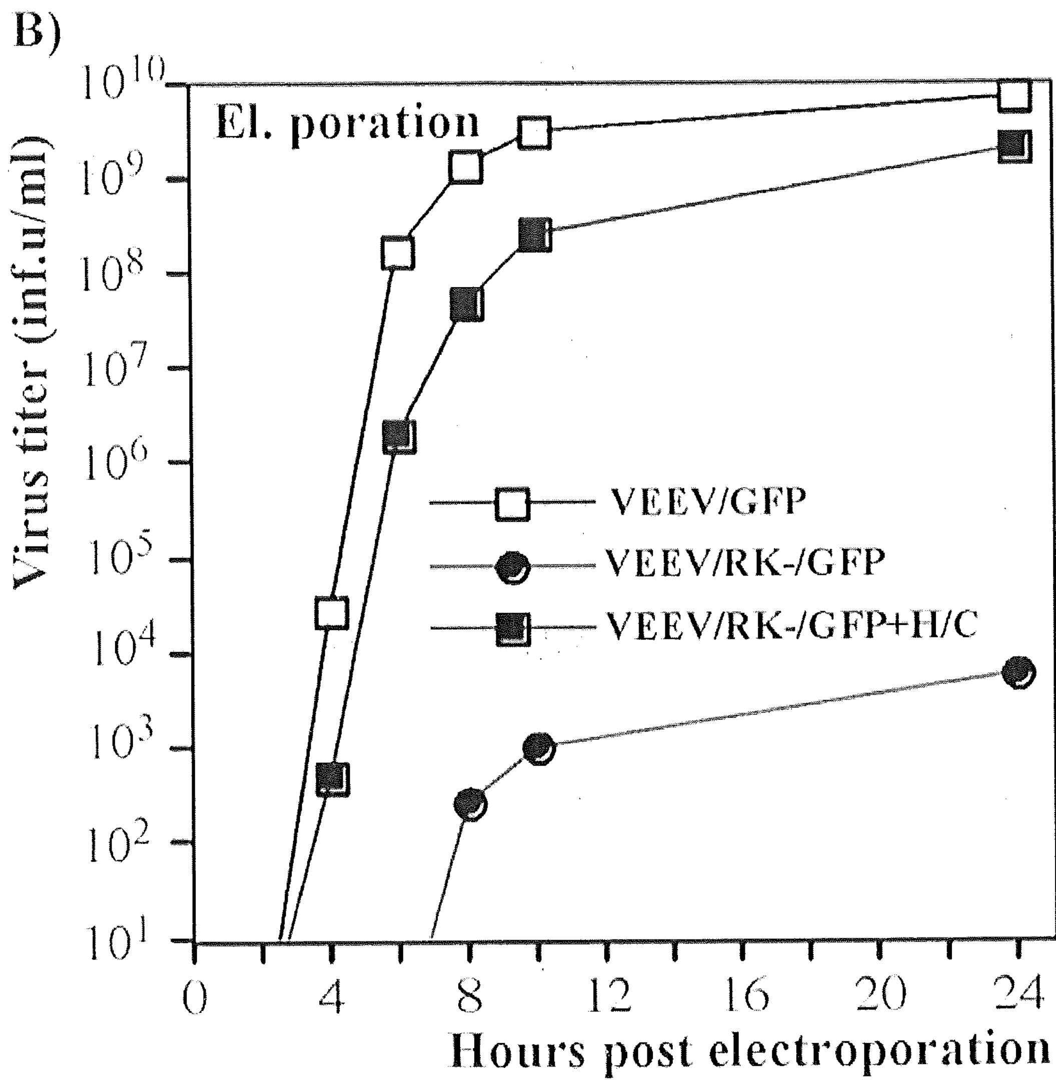
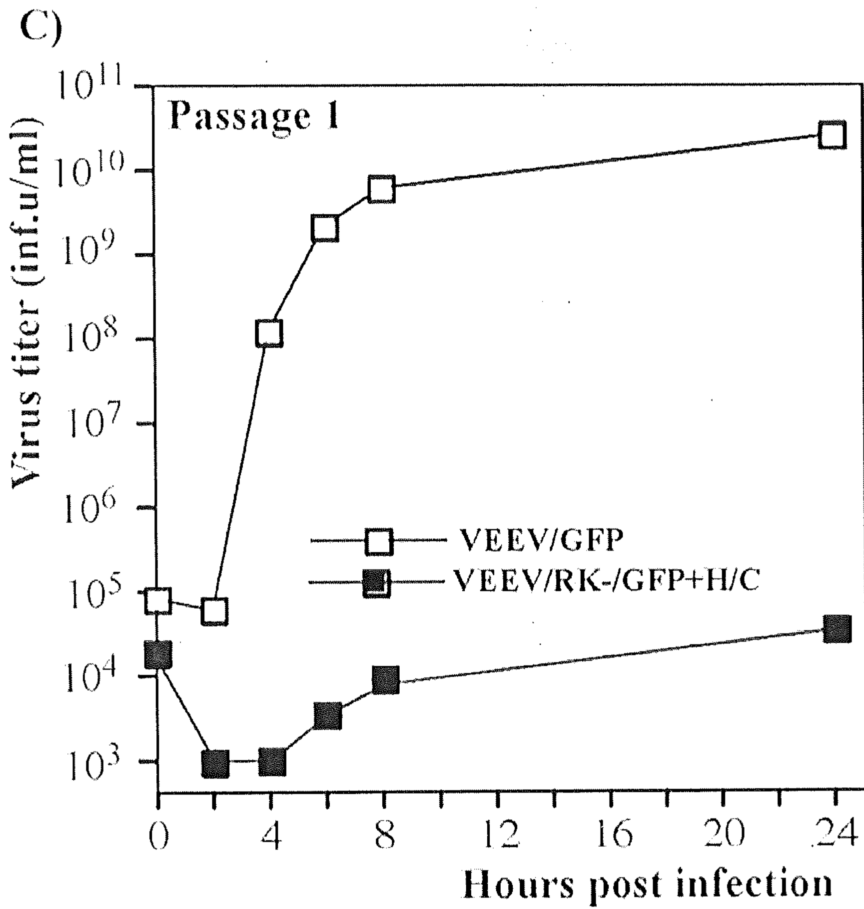


Fig. 4 (Cont.)

C)



D)

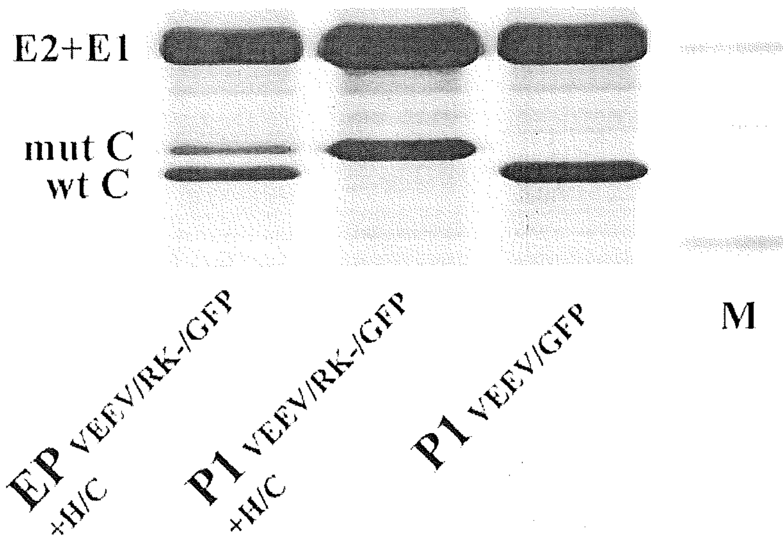


Fig. 4 (Cont.)

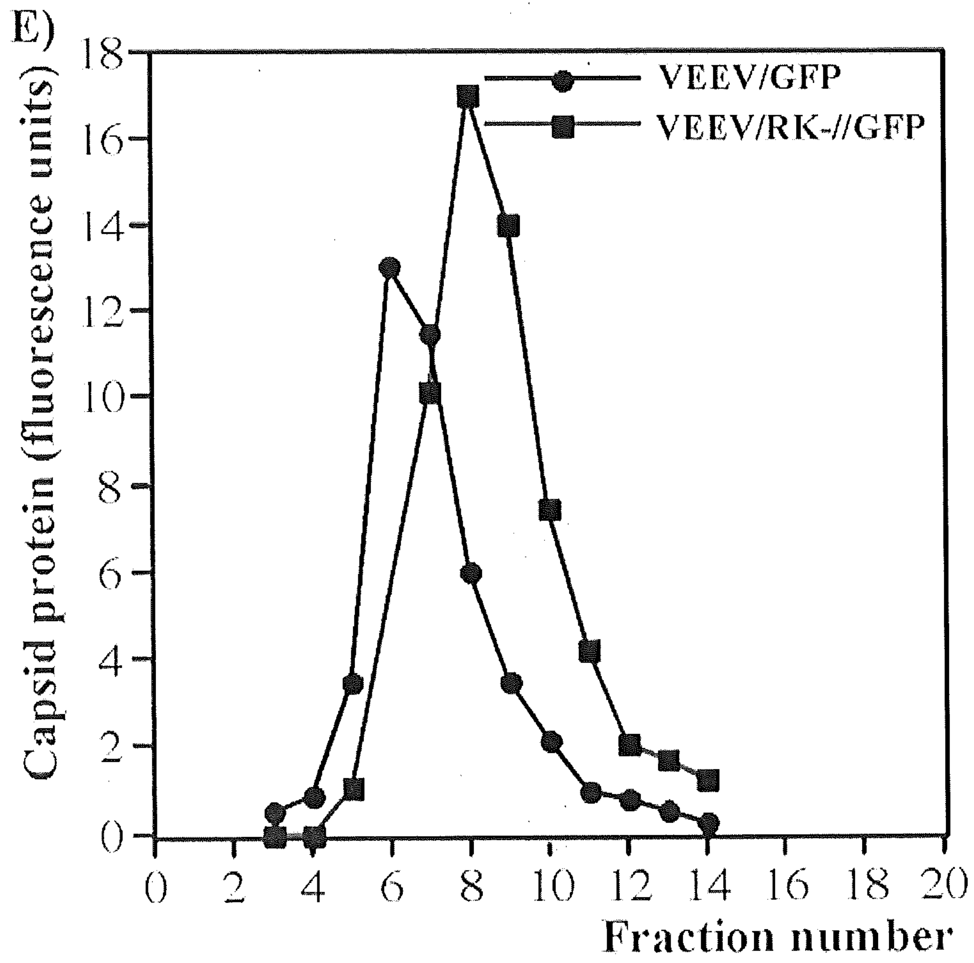


Fig. 5

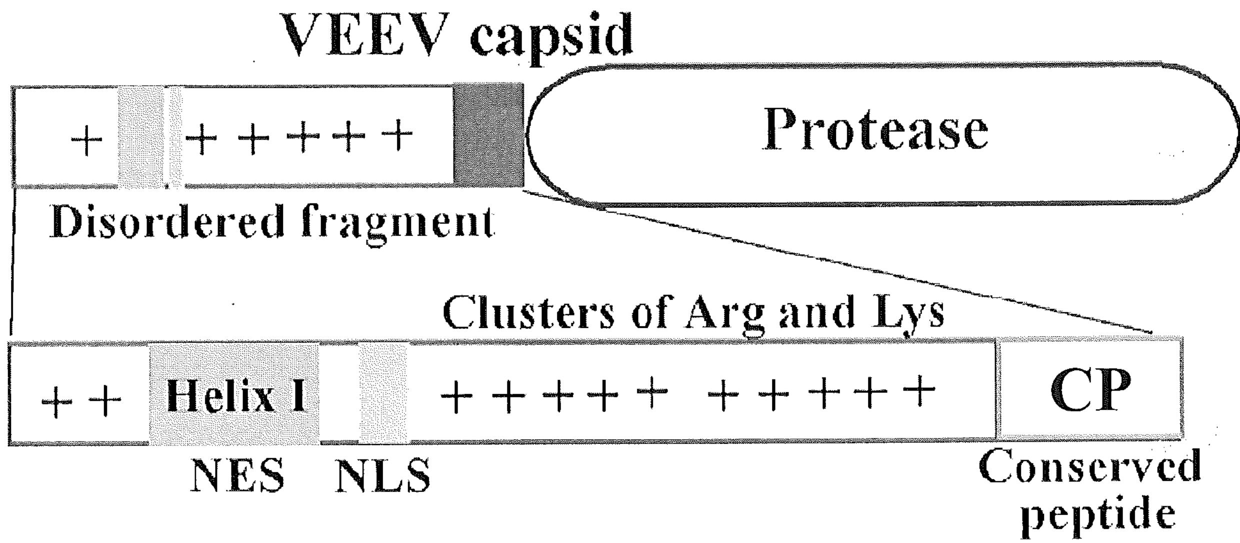


Fig. 6

Amino acid sequence of capsid protein of VEEV TC-83 (which is the same in VEEV TRD):
MFPFQPMYPMQPMPYRNPFAAPRRPWFPRTDPFLAMQVQELTRSMANLTF
KQRRDAPPEGPSAKKPKKEASQKQKGGGQGKKKKNQGKKKAKTGPPNPKA
QNGNKKKTNKKPGKRQRMVMKLESDKTFPIMLEGKINGYACVVGGKLFRP
MHVEGKIDNDVLAALKTKKASKYDLEYADVPQNMRADTFKYTHEKPQGYY
SWHHGAVQYENGRFTVPKGVGAKGDSGRPILDNQGRVVAIVLGGVNEGSR
TALSVVMWNEKGVTVKYTPENCEQW

Amino acid sequence of capsid protein of Chikungunya Thai strain:
MEFIPTQTFYNRRYQPRPWTPRSTIQIIRPRPRPQRQAGQLAQLISAVNKLTMRAVPQ
QKPRRNRKNKKQKQKQQAPQNNTNQKKQPPKKKPAQKKKKPGRRERMCMKIEND
CIFEVKHEGKVTGYACLVGDKVMKPAHVKGTIDNADLAKLAFKRSSKYDLECAQIP
VHMKSDASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGRV
VAIVLGGANEGARTALSVVTWNKDIVTKITPEGAEEW

Amino acid sequence of capsid protein of EEEV North America Florida 93:
MFPYPTLNYPPMAPINPMAYRDPNPPRRRWRPFRPPLAAQIEDLRRSIANLTLKQRAP
NPPAGPPAKRKKPAPSLSLRRKKKRPPPPAKKQKRKPKPGKRQRMCMKLESDKTFPI
MLNGQVNGYACVVGGRVFKPLHVEGRIDNEQLAAIKLKKASIYDLEYGDVPQCMK
SDTLQYTSDKPPGFYNWHHGAVQYENNRFTVPRGVGGKGDSGRPILDNKGRVVAIV
LGGVNEGSRTALSVVTWNQKGVTVKDTPEGSEPW

Amino acid sequence of WEEV McMillan strain:
MFPYPQLNFPPVYPTNPMAYRDPNPPRRRWRPFRPPLAAQIEDLRRSIAN
LTFKQRSPNPPPGPPPKKKKSAPKPKPTQPKKKKQQAKKTKRKPKPGKRQRMCMKL
ESDKTFPIMLNGQVNGYACVVGGRLMKPLHVEGKIDNEQLAAVKLKKASMYDLEY
GDVPQNMKSDTLQYTSDKPPGFYNWHHGAVQYENGRFTVPRGVGGKGDSGRPILD
NRGRVVAIVLGGANEGTRTALSVVTWNQKGVTIRDTPEGSEPWS